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TITLE: Identification of HP1 Target Genes Involved in Progression of Breast Cancer

PRINCIPAL INVESTIGATOR: Naoko Tanese, Ph.D.

CONTRACTING ORGANIZATION: New York University School of Medicine
New York, NY 10016

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6. AUTHOR(S)

Naoko Tanese, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New York University School of Medicine
New York, NY 10016

E-Mail: tanesn01@med.nyu.edu

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13. ABSTRACT (Maximum 200 Words)

Heterochromatin protein 1 alpha (HP1α) mRNA and protein have previously been reported to be decreased in the invasive, highly metastatic breast cancer cell lines, MDA-MB-231 and Hs578T. Additionally, the introduction of HP1α protein into MDA-MB-231 led to decreased metastatic potential. We hypothesized that the decrease in HP1α might be due to dysregulation of HP1α gene transcription. To test this hypothesis, we first isolated a genomic DNA fragment containing the HP1α gene promoter. Portions of the promoter sequence were transfected into stage-specific breast carcinoma cell lines. The HP1α promoter was less active in the highly metastatic breast cancer cell line MDA-MB-231 compared with other cell lines. Immunoblotting of cell lysates showed that MDA-MB-231 contained decreased HP1α protein as compared with less invasive lines. Another metastatic cell line with decreased HP1α protein levels, Hs578T, did not show a decrease in promoter activity. Transfection of siRNA against HP1α successfully reduced endogenous HP1α in two human cell lines.

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INTRODUCTION

Breast cancer arises through a complex multi-step pathway involving the accumulation of genetic alterations. A recent study found loss of expression of heterochromatin protein 1 (HP1α) in a highly invasive/metastatic breast cancer cell line (MDA-MB-231), as well as in metastatic tumor tissues from breast cancer patients [1]. Overexpression of HP1α in MDA-MB-231 cells decreased their ability to invade the extracellular matrix (ECM) *in vitro*, suggesting that reduction of HP1α is associated with tumor progression. The HP1 proteins (α, β, γ) are non-histone chromatin-associated factors that participate in gene silencing [2]. HP1 shares a domain with other polycomb group (PcG) proteins that form a large complex and repress gene transcription. Significantly, in hormone-refractory, metastatic prostate tumors, a PcG protein was found to be overexpressed, implicating this repressor in the progression of prostate cancer [3]. We hypothesize that alterations in the levels of transcriptional repressors contribute to aberrant expression of genes leading to tumor metastasis. Despite its potentially widespread role in gene silencing, genes regulated by HP1α as well as regulation of HP1α gene itself remain largely unknown. As a first step towards understanding the molecular basis of breast cancer metastasis, we have begun to characterize the regulation of HP1α gene expression in breast cancer cell lines.

BODY

Isolation of genomic DNA containing regulatory sequences for the HP1α gene

To identify the genomic sequence containing the HP1α gene promoter, the sequence of the HP1α gene and its ortholog in mice and rats were aligned with the UCSC Genome Browser [http://genome.ucsc.edu]. We found a high degree of sequence conservation throughout the two exons as well as a large portion of the non-coding intergenic regions. These conserved intergenic regions likely serve as the gene regulatory sequence for HP1α gene transcription. A short distance upstream of the HP1α coding sequence lies a gene encoding heterogeneous nuclear ribonucleoprotein A1 (hNRPA1), that is transcribed in the opposite direction. It is likely that HP1α and hNRPA1 share regulatory sequences within the 597 bp region between them (Figure 1). hNRPA1 protein has been implicated in pre-mRNA processing, mRNA metabolism, and RNA export into the cytoplasm [4].

To study the transcription of the HP1α gene in breast cancer cell lines, primers were designed to amplify the promoter region of HP1α. Two promoter fragments were designed, Clone 51, 594 bp in length that contained the sequences upstream of the putative transcriptional start site, and a shorter promoter construct Clone 52, 283 bp in length (Figure 1). Previous attempts to amplify fragments using PCR with total human genomic DNA were unsuccessful. Instead, we used a bacterial artificial chromosome (BAC RPCI 11 968 A15) that contained the desired genomic sequence as a template and successfully amplified the desired promoter DNA fragments, which were sub-cloned into the luciferase reporter plasmid pGL3-bx (Promega).

HP1α promoter activity in different human cell lines

To examine transcription of the HP1α gene in breast cancer cells, various promoter constructs were introduced into cultured mammalian cell lines (Table 1, Table 2) by transient transfection. Interestingly, both Clone 51 and 52 HP1α promoter sequences were found to be very active in all cell lines tested, as active or more active compared with the strong viral SV40 promoter, which was used as a positive control. In human cervical carcinoma cell line HeLa, Clone 51 was approximately 500-fold more active over the negative control pGL3-bx (Figure 2). Clone 52 was 120-fold more active and SV40 showed 180-fold increase in promoter activity over pGL3-bx. The activity from Clone 2.7 that contained 2.7 kb of genomic DNA downstream of the transcriptional start site (Figure 1), was marginal at 5-fold. MCF-7 cells, a non-invasive/non-metastatic breast cancer cell line, showed both Clone 51 and 52 activity to be less than that of SV40 promoter activity (Figure 3). T47D, also a non-invasive/non-metastatic breast cancer cell line, showed similar results to that of HeLa cells, with Clone 51 activity greater than that of SV40, and Clone 52 activity comparable or slightly less than that of SV40 (Figure 4). BT474 cells, a metastatic cell line, consistently showed greater activity for Clone 51 and 52 promoter fragments over SV40 (Figure 5).

In regard to the invasive/metastatic cell line, Hs578T, we found that both of the promoter fragments, Clone 51 and 52, had significantly higher activity (approximately 5-fold) than the positive control SV40 (Figure 6). The cell line with the highest metastatic potential, MDA-MB-231 cells, malignant cells from a pleural metastatic site, showed a slight decrease in promoter activity for both Clone 51 and 52 promoter fragments as compared with SV40 (Figure 7).

These results suggest that there is a decrease in the promoter activity of the HP1α gene in the MDA-MB-231 and MCF7 cell lines. They also show a relatively equal transcriptional activity between the 51 and 52 constructs. This would imply that the main transcriptional stimulatory sequence for the HP1α gene is located within the 300 bp upstream of the HP1α transcriptional start site, although some cell lines showed more activity with the entire promoter sequence. Additionally, the relative lack of activity in the 2.7 construct may be due to inhibitory factors that bind within the hNRPA1 gene or in the first intron of HP1α. It is also possible, and more likely, that the transcripts initiating from this construct are not correctly processed to generate a functional mRNA encoding for the luciferase gene product.

HP1α protein expression in various cell lines

Kirschmann et al. found HP1α protein expression to be decreased in both Hs578T and MDA-MB-231 cell lines [1]. To compare the levels of HP1α protein in different cell lines, we carried out immunoblotting experiments with whole cell lysates and anti-HP1α, β, and γ antibodies. We found that HeLa and human embryonic kidney 293 cells express approximately equal amounts of HP1α, β, and γ proteins (Figure 8). MDA-MB-231 cells produced the least amount of HP1α, and Hs578T also had little HP1α protein (Figure 9). MCF7 cells expressed less HP1α than T47D or BT474 cells. Re-probing of the same blots with an antibody against β-tubulin confirmed equal loading of the samples. The results demonstrate the greatest reduction of HP1α protein in the most metastatic cell lines, Hs578T and MDA-MB-231. Previous studies reported mRNA expression of HP1β and HP1γ between cell lines to be approximately equal [1]. We found that MCF7 and T47D cells expressed slightly less HP1β protein than BT474, MDA-MB-231, and Hs578T cell lines (Figure 9). All cell lines produced approximately equal levels of HP1γ protein.

Down-regulation of HP1α could be due to silencing of the HP1α locus. If this were true, hNRPA1, which is located very near to the HP1α gene, would also be down-regulated if the entire locus was inaccessible to transcription machinery. To test this, we performed western blot analysis to examine hNRPA1 protein levels (Figure 10). We found that hNRPA1 is expressed equally between cell lines. These results suggest that locus silencing does not play a role in the decreased expression of HP1α in the metastatic cell lines.

Endogenous HP1α expression can be reduced with siRNA

To test the hypothesis that decreased levels of HP1α lead to increased invasive potential, we attempted to knock-down endogenous HP1α mRNA by RNA interference [5]. We used two siRNAs targeting different regions of the HP1α gene, and both siRNAs successfully reduced HP1α protein levels when transfected into HeLa cells or MCF7 cells, without affecting HP1β or HP1γ protein (Figure 11 and data not shown). These results show that it is possible to knock-down HP1α in the poorly-invasive/non-metastatic cell line, MCF7. We are now in the position to test the hypothesis that decreased HP1α may lead to increased invasiveness by using a cell invasion assay (a collagen IV/laminin/gelatin matrix). The next experiment is to carry out DNA microarray analysis on cells whose endogenous HP1α has been knocked-down with siRNA to identify target genes of HP1α and to determine whether HP1α protein levels correlate with cell invasiveness.

Table 1. Cell lines used in this study

Cell Line	Organism	Cell Origin
HeLa	Human	cervical carcinoma
293	Human	embryonic kidney
MCF7	Human	stage 1 breast cancer
T47D	Human	stage 1 breast cancer
BT474	Human	stage 4 breast cancer
Hs578T	Human	stage 4 breast cancer
MDA-MB-231	Human	pleural metastases of breast cancer

Table 2. Invasive potential of human breast carcinoma cell lines
(*In vitro* invasive potential percentage was determined by invasion through a barrier of human laminin/collagen IV/gelatin matrix [6, 7].)

Cell line	Invasive Potential %	Invasiveness
MCF7	2.5 +/- 0.3	poorly-invasive
T47D	2.9 +/- 0.4	poorly-invasive
BT474	not reported	invasive/metastatic
Hs578T	14.3 +/- 0.9	invasive/metastatic
MDA-MB-231	19.3 +/- 2.0	invasive/metastatic

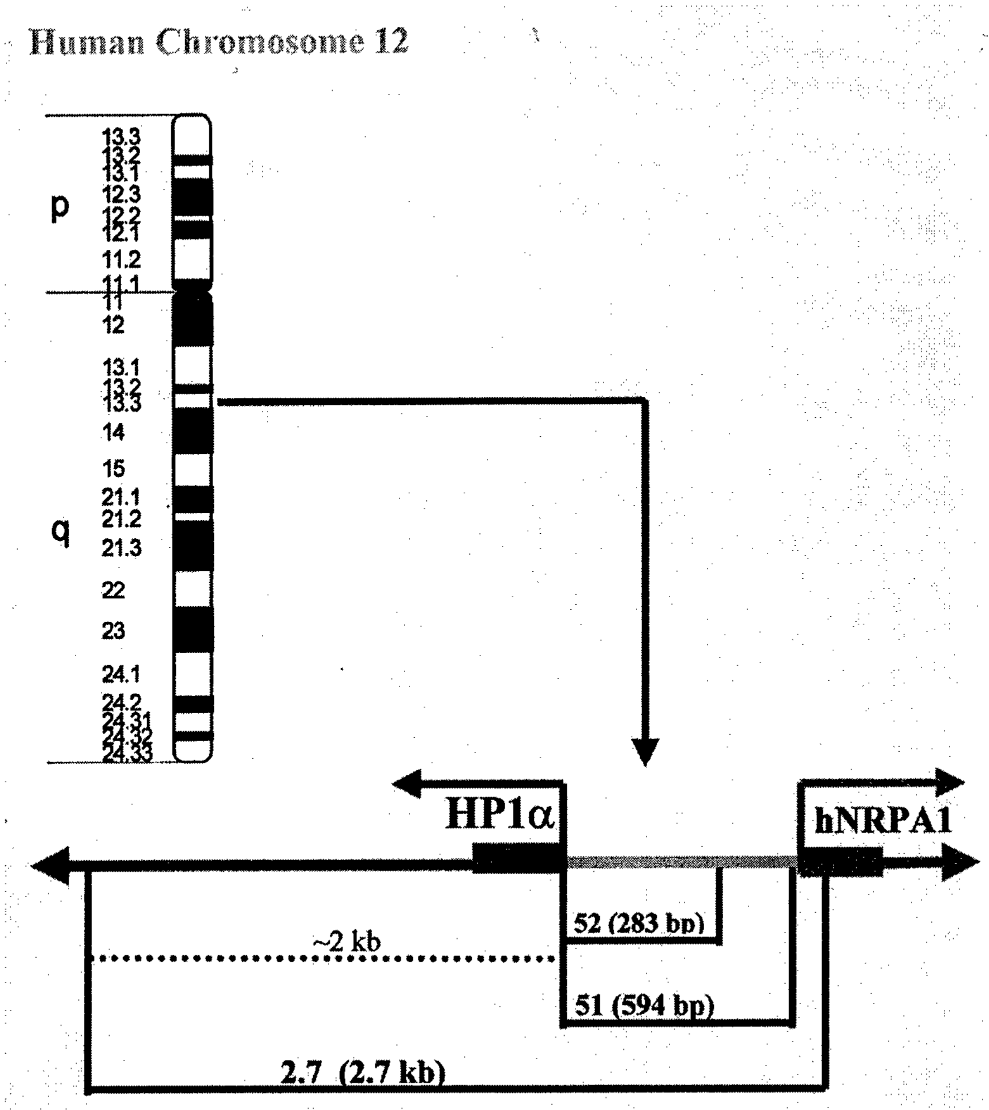
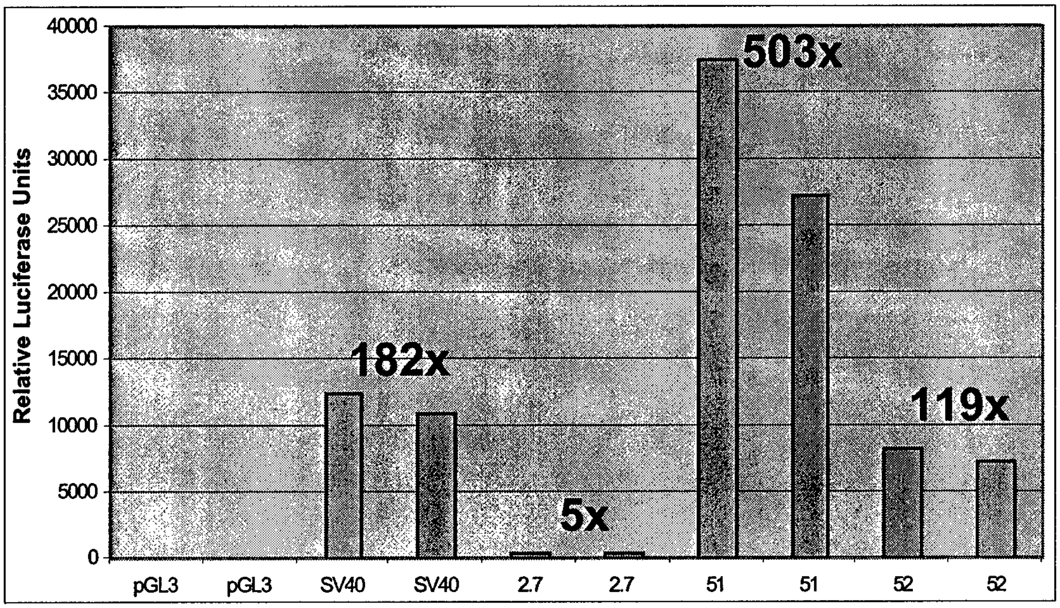


Figure 1. A schematic diagram depicting the HP1α and hNRPA1 genes. Relative location of the first exons of the HP1α and hNRPA1 genes on human chromosome 12 is shown. The three promoter constructs Clone 51 (594 bp), 52 (283 bp), and 2.7 (2.7 kb) used in this study are indicated.

A.



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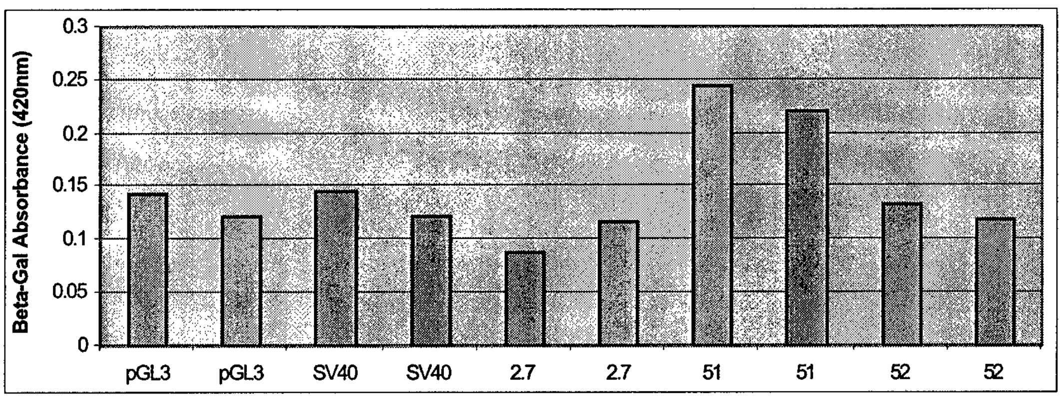
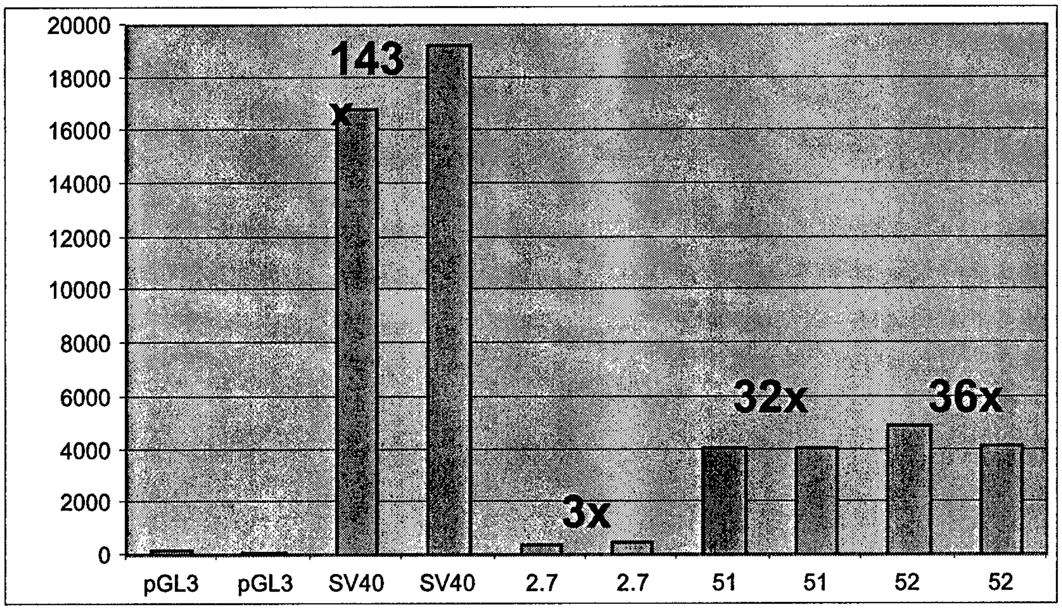


C.

	Exp 1	**Exp 2**	**Exp 3**
	$CaPO_4$	$CaPO_4$	$CaPO_4$
pGL3	1	1	1
SV40	182	40	55
2.7	5	3	3
51	503	67	102
52	119	28	69

Figure 2. HeLa cells (200,000) were transfected in duplicate with 0.5 μg of Clones 2.7, 51, 52, negative control pGL3, or positive control SV40, and 0.1 μg of CMV-LacZ using the calcium phosphate precipitation method. Cells were harvested 48 hrs later and assayed for **A)** luciferase activity and **B)** β-galactosidase activity. The numbers above the bars represent fold increase in luciferase activity as compared with pGL3 (average of two). **C.** Summary of three HeLa cell transfection experiments. The numbers represent fold increase in luciferase activity as compared with pGL3 (average of two transfections per experiment).

A.



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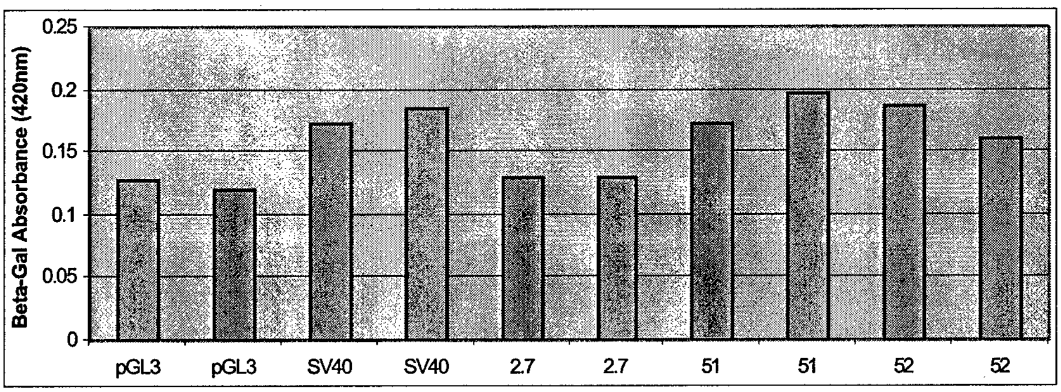
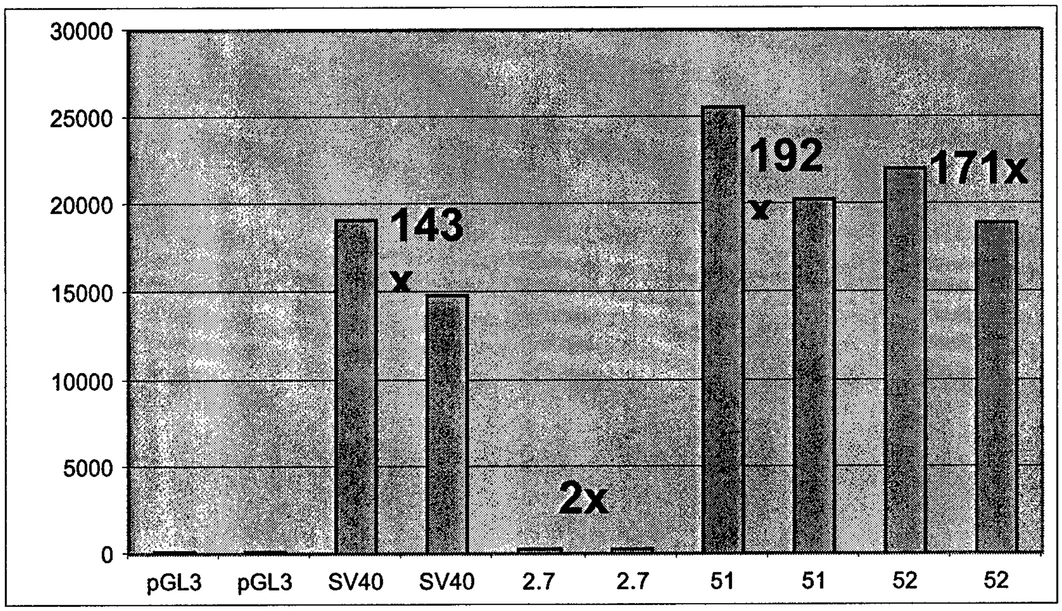


C.

	Exp 1	Exp 2	Exp 3
	Lipofectamine	Lipofectamine	Lipofectamine
pGL3	1	1	1
SV40	74	47	143
2.7	2	1.5	3
51	98	25	32
52	119	23	36

Figure 3. MCF7 cells (200,000) were transfected in duplicate with 0.5 μg of Clones 2.7, 51, 52, negative control pGL3, or positive control SV40, and 0.5 μg of CMV-LacZ using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hrs later and assayed for **A)** luciferase activity and **B)** β-galactosidase activity. The numbers above the bars represent fold increase in luciferase activity as compared with pGL3 (average of two). **C.** Summary of three MCF7 cell transfection experiments. The numbers represent fold increase in luciferase activity as compared with pGL3 (average of two transfections per experiment).

A.



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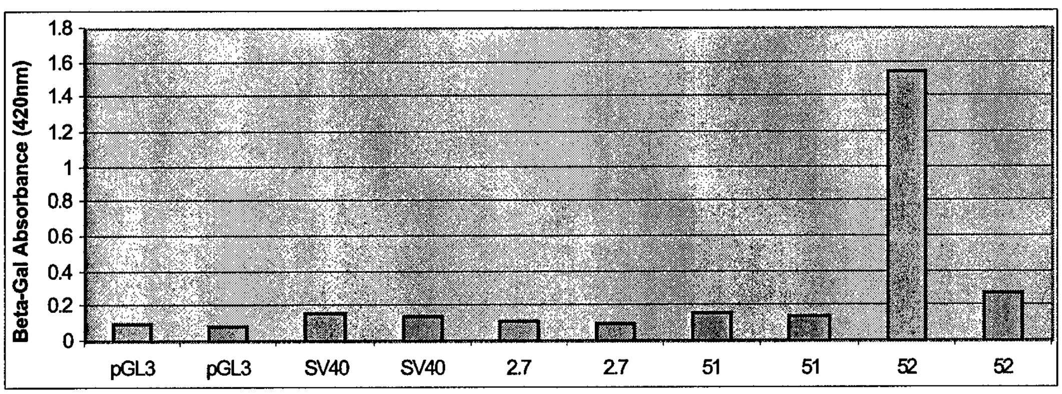
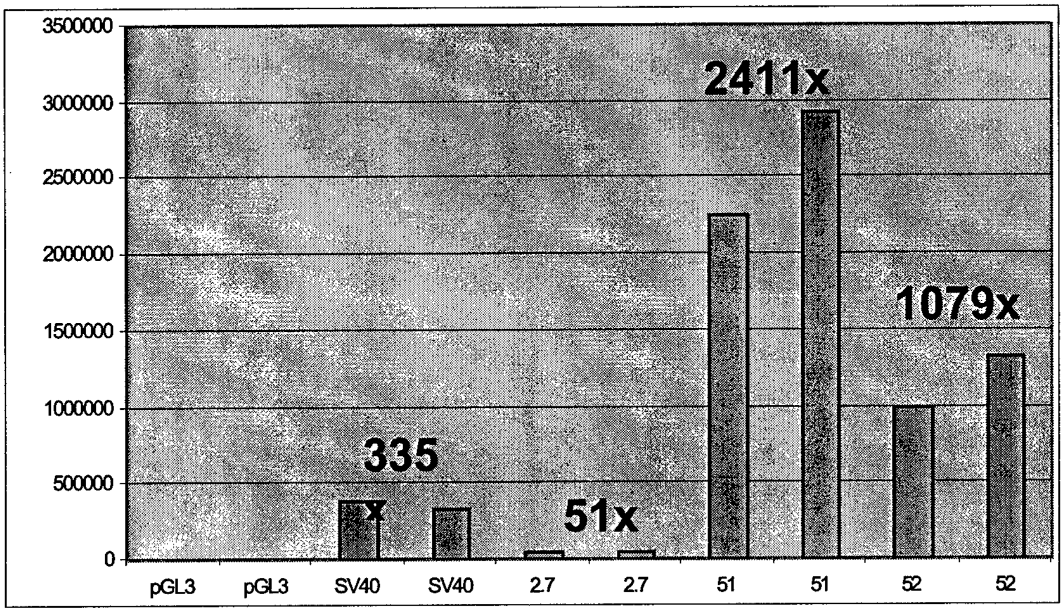


C.

	Exp 1	Exp 2
	Lipofectamine	Lipofectamine
pGL3	1	1
SV40	143	44
2.7	2	1
51	192	74
52	171	33

Figure 4. T47D cells (200,000) were transfected in duplicate with 0.5 μg of Clones 2.7, 51, 52, negative control pGL3, or positive control SV40, and 0.5 μg of CMV-LacZ using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hrs later and assayed for **A**) luciferase activity and **B**) β-galactosidase activity. The numbers above the bars represent fold increase in luciferase activity as compared with pGL3 (average of two). **C.** Summary of two T47D cell transfection experiments. The numbers represent fold increase in luciferase activity as compared with pGL3 (average of two transfections per experiment).

A.



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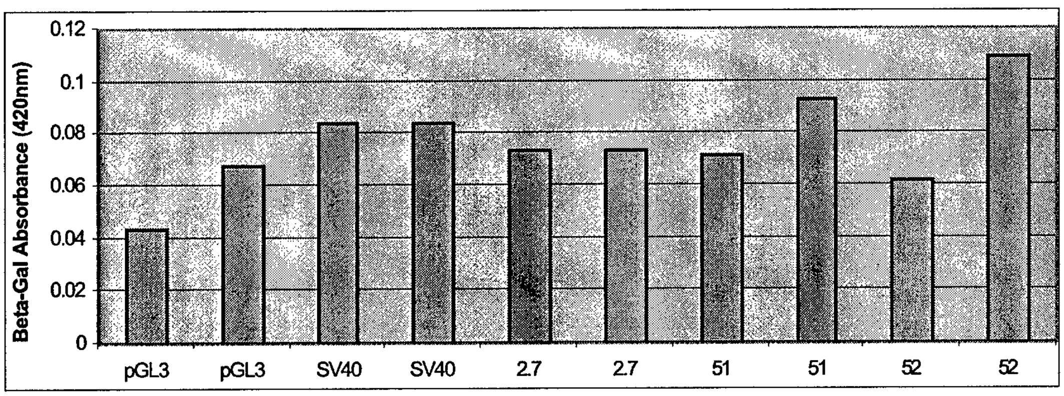
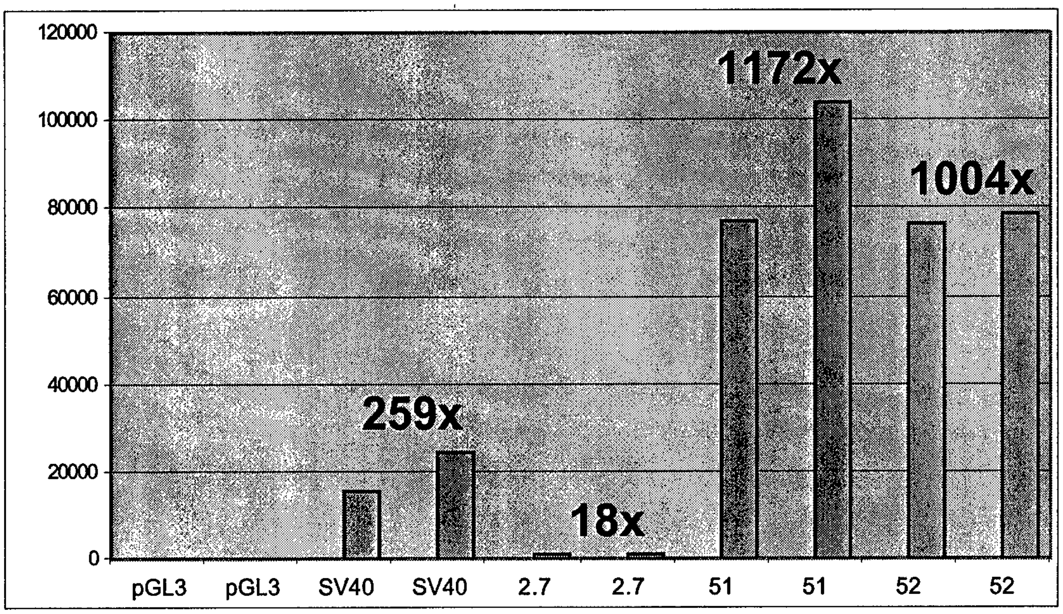


C.

	Exp 1	Exp 2
	Lipofectamine	FuGENE 6
pGL3	1	1
SV40	78	335
2.7	3	51
51	218	2411
52	153	1079

Figure 5. BT474 cells (200,000) were transfected in duplicate with 0.5 µg of Clones 2.7, 51, 52, negative control pGL3, or positive control SV40, and 0.5 µg of CMV-LacZ using FuGENE 6 (Roche). Cells were harvested 48 hrs later and assayed for **A**) luciferase activity and **B**) β-galactosidase activity. The numbers above the bars represent fold increase in luciferase activity as compared with pGL3 (average of two). **C.** Summary of two BT474 cell transfection experiments. The numbers represent fold increase in luciferase activity as compared with pGL3 (average of two transfections per experiment).

A.



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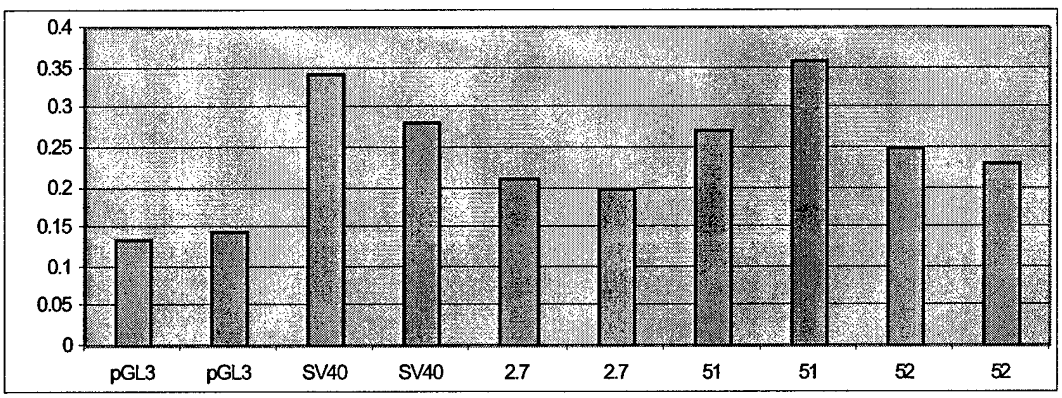
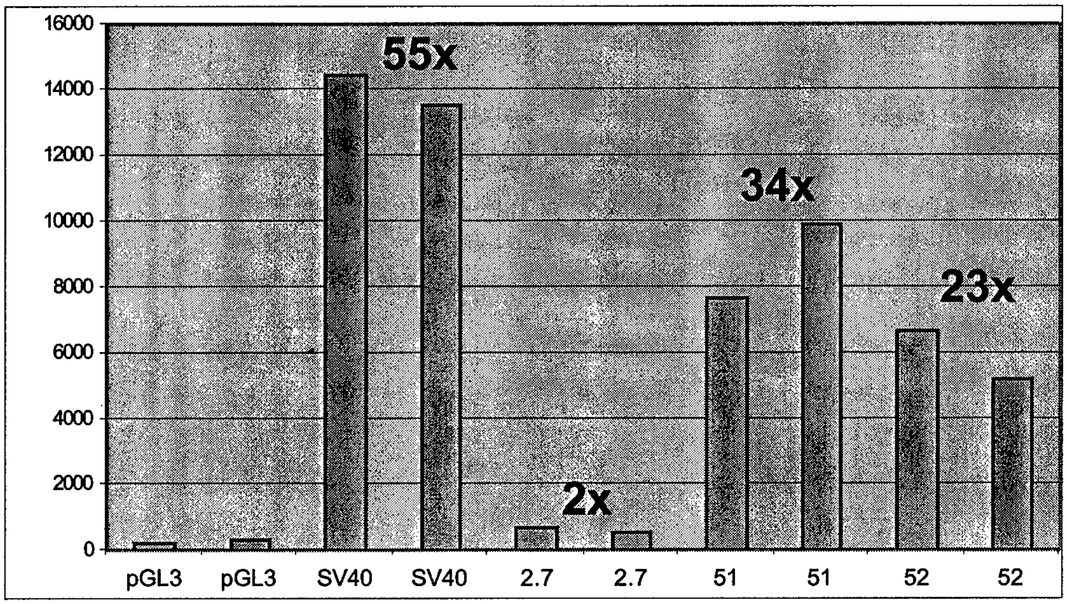


C.

	Exp 1	Exp 2
	FuGENE 6	Lipofectamine
pGL3	1	1
SV40	152	259
2.7	22	18
51	698	1172
52	1035	1004

Figure 6. Hs578T cells (200,000) were transfected in duplicate with 0.5 μg of Clones 2.7, 51, 52, negative control pGL3, or positive control SV40, and 0.5 μg of CMV-LacZ using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hrs later and assayed for **A)** luciferase activity and **B)** β-galactosidase activity. The numbers above the bars represent fold increase in luciferase activity as compared with pGL3 (average of two). **C.** Summary of two Hs578T cell transfection experiments. The numbers represent fold increase in luciferase activity as compared with pGL3 (average of two transfections per experiment).

A.



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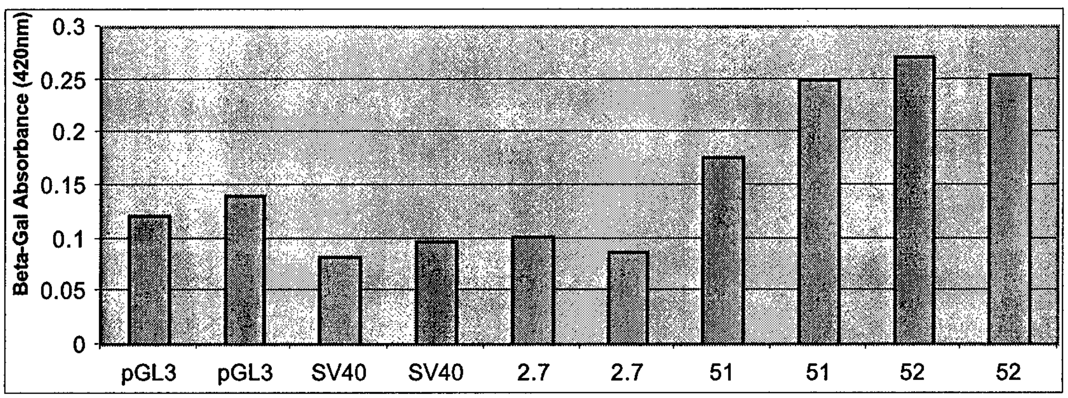

C.

	Exp 1	**Exp 2**	**Exp 3**
	Lipofectamine	FuGENE 6	FuGENE 6
pGL3	1	1	1
SV40	66	39	55
2.7	8	2	2
51	69	26	34
52	87	24	23

Figure 7. MDA-MB-231 cells (200,000) were transfected in duplicate with 0.5 μg of Clones 2.7, 51, 52, negative control pGL3, or positive control SV40, and 0.5 μg of CMV-LacZ using FuGENE (Roche). Cells were harvested 48 hrs later and assayed for **A**) luciferase activity and **B**) β-galactosidase activity. The numbers above the bars represent fold increase in luciferase activity as compared with pGL3 (average of two). **C.** Summary of three MDA-MB-231 cell transfection experiments. The numbers represent fold increase in luciferase activity as compared with pGL3 (average of two transfections per experiment).

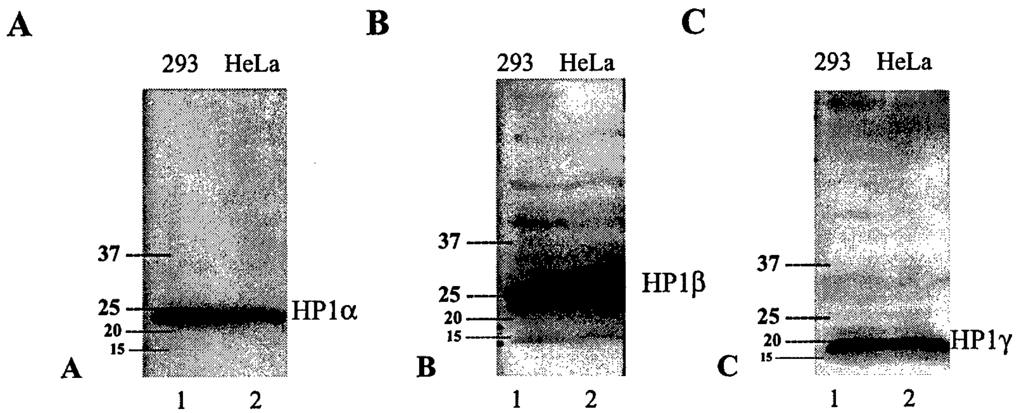

Figure 8: Western blot analysis of whole cell extracts of 293 and HeLa cells separated by SDS-PAGE and probed with the following primary antibody: **A)** anti-HP1α, **B)** anti-HP1β, **C)** anti-HP1γ. Lane 1: 293 cell lysate, Lane 2: HeLa cell lysate.

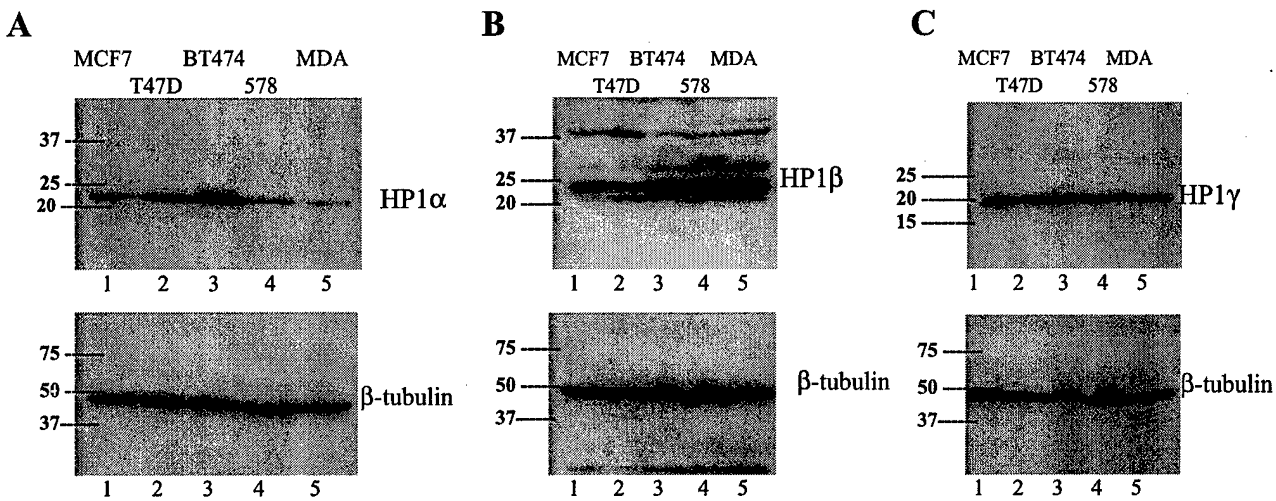

Figure 9: Western blot analysis of whole cell lysates of MCF7, T47D, BT474, Hs578T, and MDA-MB-231 cells, normalized for cell number, lysed and proteins separated by SDS-PAGE. Lane 1: MCF7; Lane 2: T47D; Lane 3: BT474; Lanes 4: Hs578T; Lane 5: MDA-MB-231 cell lysates. Upper blot was probed with: **A)** anti-HP1α, **B)** anti-HP1β, **C)** anti-HP1γ antibody. Lower blots were probed with anti-β-tubulin antibody.

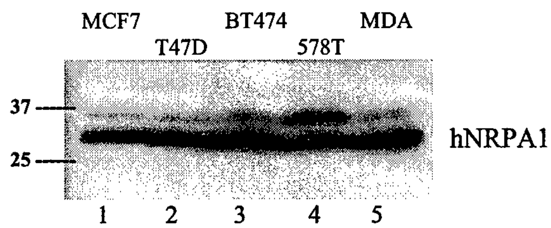

Figure 10: Western Blot analysis of whole cell lysates of MCF7, T47D, BT474, Hs578T, and MDA-MB-231 cell lines, normalized for cell number, lysed and proteins separated by SDS-PAGE. Lane 1: MCF7; Lane 2: T47D; Lane 3: BT474; Lane 4: Hs578T; Lane 5: MDA-MB-231 cell lysates. The blot was probed with anti-hNRPA1 antibody.

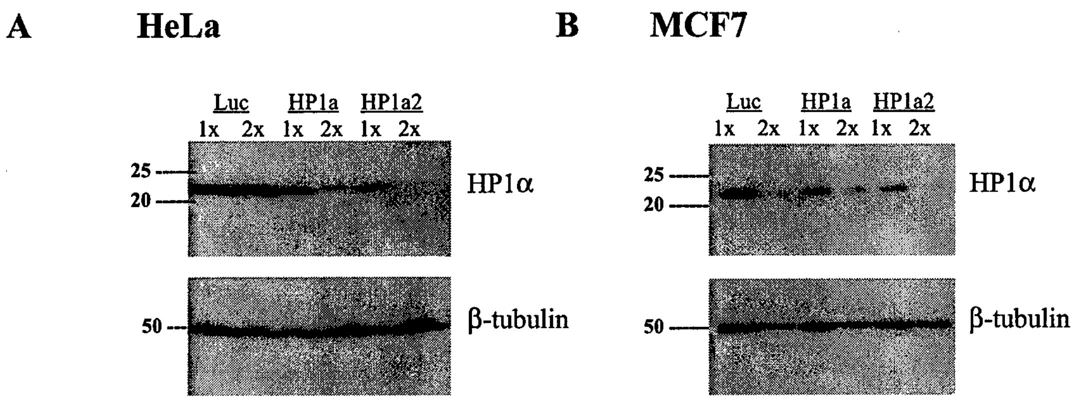

Figure 11: **A)** Western blot analysis of whole cell lysates of **A)** HeLa and **B)** MCF7 cells transfected using LipofectAMINE 2000 with two distinct siRNAs (HP1a and HP1a2) against different regions of the HP1α coding sequence. One set of each cell transfection was re-transfected 24 hours after the first transfection (notated 2x). Cells were lysed and proteins separated by SDS PAGE. The upper blots were probed with anti-HP1α antibody and the lower blots were probed with anti-β-tubulin antibody. Luc: transfected with control luciferase siRNA; HP1a: transfected with HP1a siRNA; HP1a2: transfected with HP1a2 siRNA.

KEY RESEARCH ACCOMPLISHMENTS

- Isolation of a genomic DNA segment containing the promoter region and transcriptional regulatory sequences for the HP1α and hNRPA genes.
- The HP1α gene promoter is highly active, as active as the strong SV40 viral promoter, when introduced into culture mammalian cell lines including those derived from breast carcinomas.
- The HP1α protein levels are reduced in highly invasive/metastatic MDA-MB-231 cells compared with non-invasive MCF7 cells, whereas hNRPA protein levels remain similar between different cell lines.
- Endogenous HP1α protein expression can be reduced by RNA interference in HeLa and MCF7 cells.

REPORTABLE OUTCOMES

- A manuscript containing the new findings is currently in preparation.
- An honors thesis was prepared and presented to a committee by Marissa Kaminsky. An M.D. degree with Honors was awarded to Marissa Kaminsky.
- A high school student, David Lam, entered the Intel Science Talent Search with the project in which he cloned the HP1α gene promoter.

CONCLUSIONS

HP1α mRNA and protein levels were reported to be decreased in the more invasive, highly metastatic breast cancer cell lines, MDA-MB-231 and Hs578T [1]. Additionally, the introduction of HP1α protein into MDA-MB-231 has led to decreased metastatic potential [1]. We hypothesized that the decrease in HP1α might be due to dysregulation of HP1α gene transcription. We isolated a genomic DNA fragment containing the transcriptional regulatory sequences for the HP1α gene. We transfected cell lines with portions of the HP1α promoter sequence and monitored differential promoter activity in stage-specific breast carcinoma cell lines. The HP1α promoter was highly active in all cell lines tested, although it was less active in the highly metastatic breast cancer cell line, MDA-MB-231 and the non-invasive cell line MCF7. The MDA-MB-231 and Hs578T cell lines had decreased HP1α protein levels as compared with less invasive cell types, although Hs578T did not show a decrease in promoter activity. Furthermore, the metastatic cell line, BT474, showed no decrease in HP1α protein or promoter activity. Because the expression of the hNRPA1 gene, which lies very close to the HP1α gene, was not down-regulated, we concluded that the locus encompassing these two genes was not broadly silenced.

To further elucidate the role of HP1α in metastatic breast cancer, we will evaluate the transcription factors involved in the regulation of the HP1α gene. Decreased transcription may result from alterations in transcription factor availability, such as an increase in a transcriptional repressor or a decrease in a transcriptional activator. The HP1α promoter sequence has multiple putative binding sites for a myriad of transcription factors. We will carry out mutational analysis to determine relative importance of the candidate transcription factor binding sites.

The highly metastatic cell line MDA-MB-231 showed decreased HP1α promoter activity. These cells contain less HP1α protein, consistent with the idea that a decrease in HP1α protein may lead to up-regulation of key target genes resulting in a more invasive breast cancer phenotype. However, in the metastatic cell line Hs578T, the HP1α promoter was very active despite reduced levels of HP1α protein. It is likely that additional regulatory sequences are required to fully recapitulate the regulation of the HP1α gene in a transient assay in cultured cells. We also cannot exclude the role of mRNA stability in the down-regulation of HP1α.

BT474, a known metastatic cell line, had a very active promoter and high levels of HP1α. Therefore, it is unlikely that HP1α is directly involved in the metastatic potential of this particular cell line. MCF7, the least invasive of the cell lines tested, expressed less HP1α than the more invasive cell lines T47D and BT474. It is well known that cancer arises through multiple mechanisms. It is feasible that the various cell lines attain different gene alterations, all leading to malignancy, and only some leading to metastasis. Therefore, the decrease in HP1α protein and mRNA in the MDA-MB-231 cell line could be significant for this cell line's metastatic potential, while other cell lines may obtain metastatic ability through other mechanisms.

We have demonstrated that endogenous HP1α can be knocked-down by siRNA in the poorly invasive MCF7 cell line. To test whether HP1α protein levels correlate with metastatic potential of the cell line, we will use MCF7 cells treated with siRNA and perform invasion assays to evaluate cell-spreading ability in basement membrane-like matrices. To identify the genes controlled by HP1α, we will carry out microarray analyses with RNA prepared from siRNA-treated MCF7 cells to determine those genes whose expression has altered following the loss of HP1α. Specifically, we will examine expression profiles of genes, such as vimentin, keratin, tissue factor, cyclins, plasminogen, and the retinoblastoma gene, that have been implicated in promoting metastases. Identification and characterization of novel HP1 target genes should further our molecular understanding of breast cancer progression and facilitate design of therapies for malignant cancers.

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